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Peripheral Demyelination and Neuropathic Pain Behavior in Periaxin-Deficient Mice

C. Stewart Gillespie,*‖ Diane L. Sherman,*‖ Susan M. Fleetwood-Walker,* David F. Cottrell,* Steven Tait,* Emer M. Garry,* Victoria C. J. Wallace,* Jan Ure,† Ian R. Griffiths,‡ Austin Smith,† and Peter J. Brophy*§

*Department of Preclinical Veterinary Sciences
University of Edinburgh
Edinburgh EH9 1QH
†Gene Targeting Laboratory
Centre for Genome Research
University of Edinburgh
Edinburgh EH9 3JQ
‡Applied Neurobiology Group
Department of Veterinary Clinical Studies
University of Glasgow
Glasgow G61 1QH
United Kingdom

Summary

The *Prx* gene in Schwann cells encodes L- and S-periaxin, two abundant PDZ domain proteins thought to have a role in the stabilization of myelin in the peripheral nervous system (PNS). Mice lacking a functional *Prx* gene assemble compact PNS myelin. However, the sheath is unstable, leading to demyelination and reflex behaviors that are associated with the painful conditions caused by peripheral nerve damage. Older $Prx^{-/-}$ animals display extensive peripheral demyelination and a severe clinical phenotype with mechanical allodynia and thermal hyperalgesia, which can be reversed by intrathecal administration of a selective NMDA receptor antagonist. We conclude that the periaxins play an essential role in stabilizing the Schwann cell–axon unit and that the periaxin-deficient mouse will be an important model for studying neuropathic pain in late onset demyelinating disease.

Introduction

The Schwann cell is the major glial cell of the vertebrate peripheral nervous system (PNS), where its prime function is to myelinate nerve fibers and to promote rapid nerve impulse transmission. Schwann cells have a significant secondary role in providing trophic support for spinal motoneurons and dorsal root ganglion neurons (Riethmacher et al., 1997) and in promoting nerve regeneration in the PNS (Bunge, 1993). Hence, these versatile cells play a vital role not only in the normal development of the PNS but also in the process of repair.

Extracellular signals, including those from the axon, are believed to be responsible for the induction of the genes required for myelination in the Schwann cell, and they are also thought to determine the thickness of the myelin sheath (Aguayo et al., 1977; Lemke and Chao, 1988; Yin et al., 1998). As in other cell types, the downstream pathways that transduce these signals are likely integrated by the cytoskeleton (Tapon and Hall, 1997) since it has an essential role in mediating both the changes in cell shape and the regulation of gene expression required for axonal ensheathment (Fernandez-Valle et al., 1997). Hence, it was of interest that biochemical analysis of the cytoskeleton-associated proteins of myelinating Schwann cells revealed periaxin as a protein with a possible role in the later stages of myelination (Gillespie et al., 1994). As myelin sheaths mature, periaxin becomes concentrated in the abaxonal plasma membrane, and this shift in localization suggested that it might participate in the recruitment of proteins to a cortical structure involved in transmembrane signaling (Scherer et al., 1995).

Consistent with a role in cortical signaling, the murine *Prx* gene encodes two proteins with PDZ domains, L-periaxin and S-periaxin, of 147 and 16 kDa, respectively (Dytrych et al., 1998). PDZ motifs are protein interaction modules of ~90 amino acids named after the three proteins in which they were first identified, namely postsynaptic density protein PSD-95, *Drosophila discs large* (*dlg*) tumor suppressor, and the tight junction-associated protein ZO-I (Kornau et al., 1995). The PDZ motif is present in a variety of proteins believed to have an organizing function at sites of cell–cell contact, where they are implicated in the assembly of macromolecular signaling complexes (Kornau et al., 1997; Tsunoda et al., 1997). Such proteins can multimerize homotypically and heterotypically (Hsueh et al., 1997; Srivastava et al., 1998). They may also interact with the cytoplasmic tail of transmembrane proteins, such as glutamate receptors (Dong et al., 1997; Sheng and Wyszynski, 1997; Srivastava et al., 1998).

The evidence so far suggests that the periaxins participate in the later stages of axonal ensheathment, possibly in the stabilization of the myelin sheath (Scherer et al., 1995). If this is correct, inactivation of the *Prx* gene should destabilize the relationship of the axon with its ensheathing Schwann cell. We have generated mice that lack a functional *Prx* gene, and we demonstrate that their Schwann cells ensheath and myelinate peripheral nerve axons in an apparently normal manner but that this sheath destabilizes, and the mice develop a severe demyelinating neuropathy. These data suggest that the periaxins play an essential role in the establishment of a stable Schwann cell–axon unit in the myelinated fibers of the vertebrate PNS.

In addition to reducing the rates of nerve impulse transmission, segmental demyelination in human disease can be associated with tactile allodynia, the perception of normally innocuous stimuli, such as touching or brushing, as painful, and hyperalgesia, a heightened response to painful stimuli; however, the mechanisms of neuropathic pain in demyelinating disease are poorly understood (Rasminsky, 1981). Importantly, we show that in addition to a marked reduction in their peripheral nerve conduction velocities, periaxin null mice display

§To whom correspondence should be addressed (e-mail: peter.brophy@ed.ac.uk).
‖These authors contributed equally to this work.

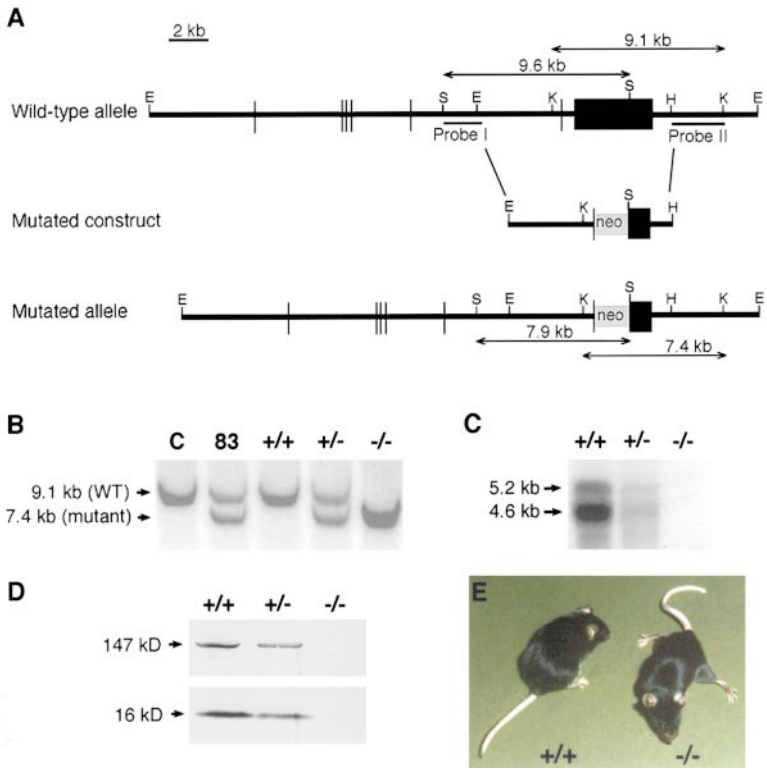

Figure 1. Targeted Disruption of the *Prx* Gene

(A) Structure of wild-type and targeted *Prx* loci.

(Top) A partial restriction endonuclease map showing the position of SacI (S) and KpnI (K) sites in the murine *Prx* gene. A β-actin promoter–*neo* gene cassette was inserted between the SmaI site in exon 6 and the SacI site in exon 7. Digestion of DNA with SacI identified the targeted allele by the presence of a 7.9 kb fragment instead of a 9.6 kb when probed with external probe I, and by a 7.4 kb instead of 9.1 kb when DNA was digested with KpnI and probed with external probe II.

(B) DNA from a control ES clone (C), from a homologous recombinant ES clone (83), and from tail clips of 3-week-old littermates obtained by heterozygous intercrosses was digested with KpnI and probed with external probe II.

(C) Northern blot analysis of RNA (3 μg) from sciatic nerves of 16-day-old $Prx^{+/+}$, $Prx^{+/-}$, and $Prx^{-/-}$ mice demonstrates the absence of the *Prx* wild-type mRNAs in mice homozygous for the mutant allele.

(D) Western blot of sciatic nerve homogenate protein (10 μg) from 1-month-old mice demonstrates the absence of both L- and S-periaxin (147 and 16 kDa, respectively) from homozygous mutants.

(E) $Prx^{-/-}$ mice at 8 months are unable to support themselves on their hindlegs.

reflex behaviors associated with allodynia and hyperalgesia. We suggest that these animals will prove to be valuable in identifying the pathophysiological basis of neuropathic pain.

Results

Generation and Clinical Phenotype of *Prx* Null Mice

To inactivate the *Prx* gene, we deleted exon 6 and part of exon 7, corresponding to 611–3440 bp of the mouse cDNA, by homologous recombination in embryonic stem (ES) cells (Figure 1A); 2 of 130 lines, clones 57 and 83, were heterozygous for insertion at the *Prx* locus, and clone 83 was selected for further study (Figure 1B). Homozygous offspring lacked the 4.6 and 5.2 kb mRNAs found in the peripheral nerves of $Prx^{+/+}$ mice (Figure 1C), and neither L- nor S-periaxin polypeptides were detectable in the peripheral nerves of mutant mice (Figure 1D). The genotypes of the progeny of mice heterozygous for the mutant allele were as expected from Mendelian laws, which indicated that embryonic development was not compromised by inactivating the *Prx* gene. Mice homozygous for the mutant allele appeared grossly normal for up to 6 weeks, with the exception that when lifted by the tail, they all displayed a distinctive clasping of the hindlimbs from about 4 to 6 weeks of age, and some animals displayed a slight tremor. By 6–9 months of age, there was a pronounced unsteadiness in the mutants' gait, and they had great difficulty supporting themselves on their hindlimbs, which were often splayed (Figure 1E). Older animals (>6–9 months) lost weight rapidly, owing to an inability to feed, and their breathing was labored. As soon as the animals were in obvious distress, they were euthanized.

Structure of the Schwann Cell–Axon Unit

We next examined the peripheral nerves of periaxin-deficient mice by light microscopy to determine if the myelin sheath was affected. At 6 weeks of age, the sciatic nerves of periaxin null mice contained focal thickenings (tomacula) and infoldings of internodal myelin (Figure 2B). Nevertheless, Western blotting showed that the levels of the major myelin proteins, myelin-associated glycoprotein, P0, and myelin basic protein, were normal in the sciatic nerves of mutant animals, which confirmed that at this age there had not been extensive demyelination (data not shown). A variety of spinal, cranial, and autonomic nerves also displayed limited abnormalities at this age. However, by 6 months, sensory, motor, and autonomic (vagus) nerves were extensively demyelinated, and most internodes in the sciatic nerves of periaxin null mice contained focal thickenings or infoldings of the sheath (Figure 2D). Profound disruption of axonal ensheathment and segmental demyelination was apparent in teased sciatic nerve fibers at this age (Figures 2E and 2F), and their Schmidt-Lanterman incisures, which are normally visible as cytoplasm-filled structures along the length of the internodes, were deranged (Figures 2G and 2H).

Electron microscopy demonstrated that saphenous nerves, which are predominantly sensory, were extensively hypermyelinated but that unmyelinated C fiber bundles appeared to be morphologically normal, which is consistent with the absence of *Prx* gene expression in

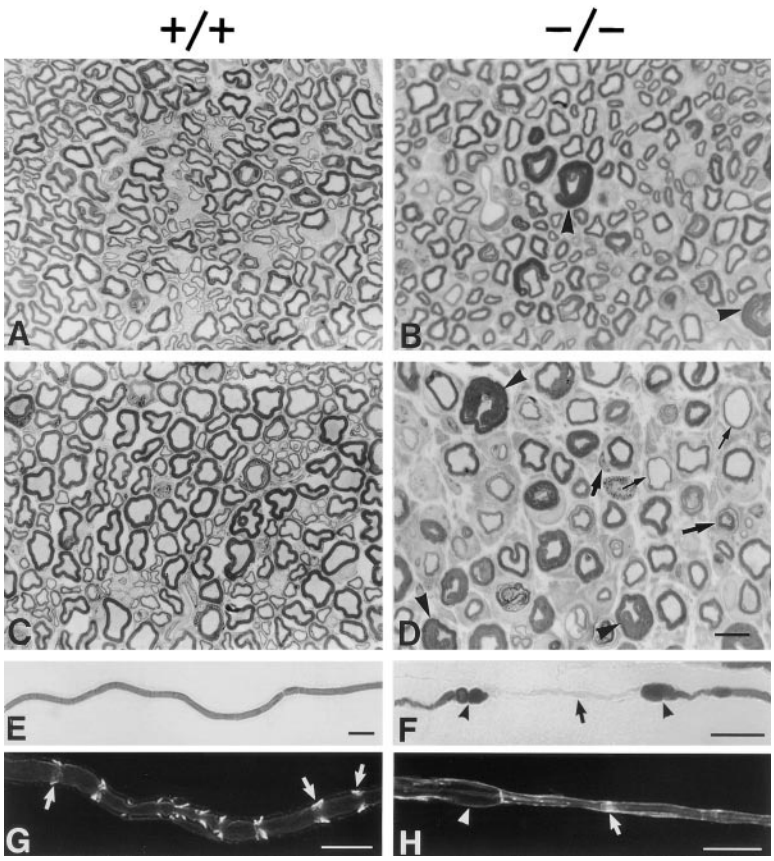

Figure 2. Light Microscopy of Sciatic Nerves from *Prx* Null Mice

Resin sections (1 μm) from the sciatic nerves of normal (A and C) and mutant (B and D) mice at 6 weeks (A and B) and at 6 months (C and D) were compared. The null mutant (B and D) showed features typical of a progressive demyelinating neuropathy with evidence of remyelination, as denoted by onion bulbs and thin myelin sheaths (thick and thin arrows, respectively). Disproportionately thick sheaths evident at 6 weeks are much more numerous at 6 months (arrowhead). A low-power view of wild-type (E) and mutant (F) teased sciatic nerve fibers from 8-month-old $Prx^{-/-}$ mice shows focal thickenings of the myelin sheath (arrowheads) flanking a demyelinated segment. A higher-power view of nerves stained with TRITC-phalloidin (G and H) reveals the loss of Schmidt-Lanterman incisures in the sheath (arrows); a tomaculum is visible in outline (arrowhead). Scale bar, 10 μm (A–D); 50 μm (E and F); 25 μm (G and H).

non-myelin-forming Schwann cells (Scherer et al., 1995) (Figures 3A–3C). Hypermyelination resulted in sheath infolding (Figure 3B) and axonal compression (Figure 3C). By 8 months, naked or thinly myelinated axons were common in sciatic nerve fibers, often surrounded by redundant basal laminae and supernumerary Schwann cells, which formed onion bulb structures, diagnostic of attempts to remyelinate demyelinated fibers (Figures 3D). There was little evidence for macrophage infiltration, indicating that demyelination was not the result of a macrophage-mediated inflammatory process. The damage seemed to be confined to the myelin sheath in that there was no evidence of axonal degeneration, and there was no difference in the number of dorsal root ganglion neurons (L5) between wild-type controls (8655 ± 127, n = 3) and periaxin-deficient mice (8668 ± 619, n = 3). Furthermore, neuronal cell bodies in spinal gray matter and spinal ganglia showed no evidence of degenerative changes (data not shown). Mice heterozygous for the null allele ($Prx^{+/-}$) showed occasional focal thickenings of the myelin sheaths, a feature that did not increase with age, at least up to 10 months, and no evidence of demyelination was noted.

The ability of periaxin-deficient mice to remyelinate was determined after crush lesion. Electron microscopy of sciatic nerves distal to the lesion site 1 month post crush revealed that 4-month-old $Prx^{-/-}$ mice differed markedly from their wild-type littermates, in spite of their being able to assemble apparently normal compact myelin in the early developing PNS. In comparison with the myelinated reinnervated axons of control nerves, remyelination was retarded in the periaxin null mice, with many incompletely ensheathed axons and abnormally thin sheaths (Figures 3E and 3F).

Peripheral Nerve Electrophysiology in $Prx^{-/-}$ Mice

At 6–8 months, the extensive pathology noted in peripheral nerves was reflected in reduced conduction velocities in nerves from control and $Prx^{-/-}$ mice. Both the peak compound action potential (CAP) and the electromyogram from single identified skeletomotoneurons of the sciatic nerve were reduced by ~60% (Table 1). The values derived from both techniques were similar, suggesting that sensory and motor branches were equally affected in this mixed nerve, a conclusion that was supported by morphological examination of the ventral and dorsal spinal roots. The spike shape of the CAP in mutants was distorted, which may reflect a degree of ephaptic cross-activation caused by extensive demyelination (Smith and McDonald, 1980) (data not shown).

It was of particular interest to analyze sensory nerves since they have been reported to be spared in animals deficient in other PNS myelin proteins (Adlkofer et al., 1995; Martini et et al., 1995; Anzini et al., 1997). The conduction velocities of hair follicle mechanoreceptor afferents in the saphenous nerve were tested by focal displacement of single identified hairs with von Frey filaments. Disruption of the myelinated fibers of these afferents in 6-week-old mutants was reflected in a significant reduction in their mean conduction velocities from 7.6 ± 3.3 (5 animals, 52 mechanoreceptors) to 3.9 ± 1.6 ms^{-1} (6 animals, 42 mechanoreceptors) ($p < 0.0001$ by

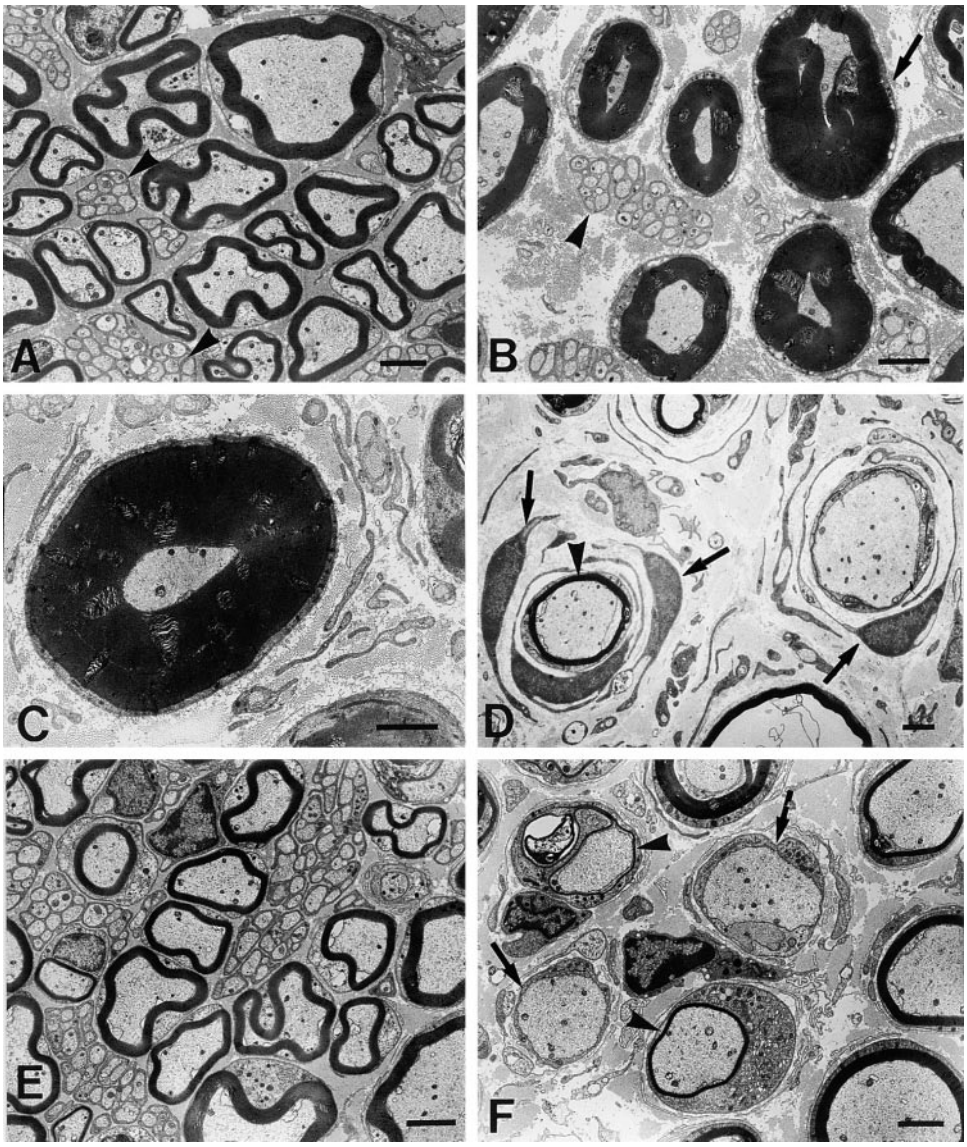

Figure 3. Structure of Schwann Cell–Axon Units in Nerves from *Prx* Null Mice

Electron microscopy of wild-type (A) and mutant (B and C) saphenous nerves at 6 months. Unmyelinated C fiber bundles appear normal (arrowhead), but myelinated fibers are grossly hypermyelinated (B and C), with evidence of myelin infolding at a tomaculum (arrow, [B]) and axonal compression (C). By 8 months, the sciatic nerves of mutant animals (D) display the onion bulb pathology of supernumerary Schwann cells (arrows) characteristic of a demyelinating neuropathy with attempts at remyelination that results in disproportionately thin myelin sheaths (arrowhead). In 4-month-old wild-type animals, robust reinnervation and remyelination were observed in sciatic nerves distal to the crush site 1 month post crush (E). In contrast, ensheathment of mutant nerves (F) was highly retarded (arrows), with numerous abnormally thin sheaths (arrowheads). Scale bars, 2 μm.

Student's t test). Only 8% of recorded afferents in mutant animals had conduction velocities >6 ms^{-1}, and none were above 8 ms^{-1}, in marked contrast to wild-type animals (Figure 4). The vast majority of these were in the A fiber conduction velocity range, with a conduction velocity above 2 ms^{-1}, indicating that they were myelinated (Koltzenburg et al., 1997); only a minor population, in each case, displayed conduction velocities characteristic of unmyelinated fibers (Figure 4). Similar results were obtained at 6 months, but the reduction in conduction velocities (67%) was more severe (data not shown).

The unmyelinated fibers were also specifically investigated in 6-week-old animals, and there was no significant difference in the mean conduction velocity in mutants compared with normal littermates (*Prx*$^{+/+}$, 1.46 $\pm$ 0.10 ms^{-1}, n = 11 afferent units; *Prx*$^{-/-}$, 1.17 $\pm$ 0.13 ms^{-1}, n = 15 afferent units; three mice in each case and $p > 0.05$ by Student's t test); nor was there a significant difference between normal and mutant mice in the von Frey filament response thresholds of the A fibers or the thermal nociceptive response thresholds of the C fiber population (Table 2).

A common feature of the saphenous nerves of both mutant animals and their wild-type littermates at 6 weeks was the presence of a spontaneous low-frequency discharge (1–2 Hz) in fibers throughout the

Table 1. Nerve Conduction Velocities in Sciatic Nerves at 8 Months of Age

	Peak CAP	EMG
Wild-type	25.0 ± 0.7	27.5 ± 1.6
Mutant	10.9 ± 2.0	8.6 ± 1.0
	$p < 0.005$	$p < 0.0001$

Compound action potential (CAP) and electromyogram (EMG) values (ms^{-1}) were measured after electrical excitation of the peripheral nerve and are mean ± SEM for four animals in each group. Statistical significance was determined by Student's t test.

range of conduction velocities. However, more mutants (100%, n = 14) displayed this activity in comparison to their wild-type counterparts (28%, n = 15). This spontaneous activity occurred across the conduction velocity range.

Peripheral Nerve Conduction and Behavioral Deficit

The spontaneous low-frequency discharge observed in saphenous fibers prompted us to analyze the response of the mice in well-characterized models of nociceptive reflex behavior since it has been suggested that ectopic activity might contribute to pain following nerve injury (Rasminsky, 1981; Tal and Eliav, 1996). At 6 weeks of age, mutant mice displayed a markedly lower threshold in a reflex test of cutaneous mechanical sensation using calibrated von Frey hairs applied to the saphenous territory of the hindpaw (Table 3). This was in spite of the fact that we had already shown that the mean threshold response of hindpaw mechanoreceptors to natural mechanical stimuli, measured electrophysiologically in the saphenous nerve at 6 weeks, was unchanged (Table 2).

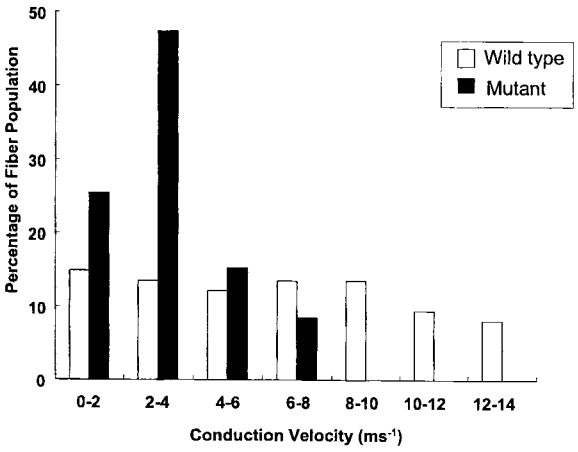

Figure 4. Frequency Range of Conduction Velocities in Saphenous Nerve Fibers

Nerve conduction velocities were measured from single afferent fibers in the saphenous nerves of 6-week-old mice. All units with conduction velocities above 2 ms^{-1} were tested and found to be mechanosensitive, and demonstrated a majority of low-threshold, rapidly adapting units, with very few slowly adapting units. The locations of peripheral receptive fields for natural mechanostimulation and electrical excitation were identical. Receptive fields were focal, confined to between one and two hairs, and afferent units were not excited when stimulating electrodes were slightly displaced from the natural receptive field.

Table 2. Threshold of the Response of Teased Saphenous Nerves to Mechanical and Thermal Stimulation with von Frey Filaments or Noxious Radiant Heat at 6 Weeks of Age

	Threshold of Activation	
	Mechanical ($mNmm^{-2}$)	Noxious Thermal (°C)
Wild-type	23.8 ± 6.6	48.2 ± 0.7
Mutant	23.4 ± 4.2	48.0 ± 0.3

Fibers tested by mechanical stimulation had conduction velocities above 2 ms^{-1} (n = 5 in both groups); those tested by noxious thermal stimulation had conduction velocities below 2 ms^{-1} (n = 3 in both groups). Each value is the mean ± SEM, and there was no statistically significant difference by Mann-Whitney Rank Sum test.

To determine if this diminished response threshold was specific to receptors with myelinated afferents, we also measured the response threshold in a reflex test of thermal nociceptive sensitivity for which the relevant afferents are largely unmyelinated C fibers (Hargreaves et al., 1988; Ahlgren et al., 1997; Koltzenburg et al., 1997). *Prx* null mice had significantly lower response thresholds, which indicated the presence of thermal hyperalgesia, as well as mechanical allodynia (Table 3). As pointed out earlier, there was no evidence for sensory neuron loss that might have led to altered reflex behavior.

In contrast to the lowered threshold in the nociceptive reflex tests, mutant mice at 6 weeks displayed responses in the normal range in a mechanical grip test (Meyer et al., 1979), which indicates a lack of any overt motor deficit at that age (data not shown).

The mechanical response thresholds (Figure 5) and nociceptive response latencies (Table 4) were also significantly reduced in 6-month-old mutants when morphological derangement of myelinated nerves was more pronounced. In these animals, a role for spinal NMDA receptors in the mechanical allodynia shown by *Prx* null mice was demonstrated by reversal of this behavioral sensitization following spinal administration of the highly selective NMDA receptor antagonist 3-([R] 2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid ([R]-CPP) (Lehmann et al., 1987) (Figure 5). Similarly, a reduction in thermal nociceptive response latency was reversed in $Prx^{-/-}$ mice without affecting the responses of normal littermates (Table 4).

Table 3. Hindpaw Withdrawal Response to Mechanical and Noxious Thermal Stimulation at 6 Weeks of Age

	Mechanical Withdrawal Threshold ($mNmm^{-2}$)	Withdrawal Latency from Noxious Heat (s)
Wild-type	460 ± 40	11.5 ± 2.2
Mutant	274 ± 23*	6.1 ± 1.1**

Minimum filament indentation pressure thresholds for repeat paw withdrawal responses to mechanical stimulation with von Frey filaments in conscious animals were measured for four animals in each group. Statistical significance (*$P < 0.05$) was determined by Mann-Whitney Rank Sum test. The time taken for hindpaw withdrawal from an infrared beam was measured for six normal littermates and seven mutant animals. Statistical significance (**$P < 0.05$) was determined by Student's t test. Each value is the mean ± SEM.

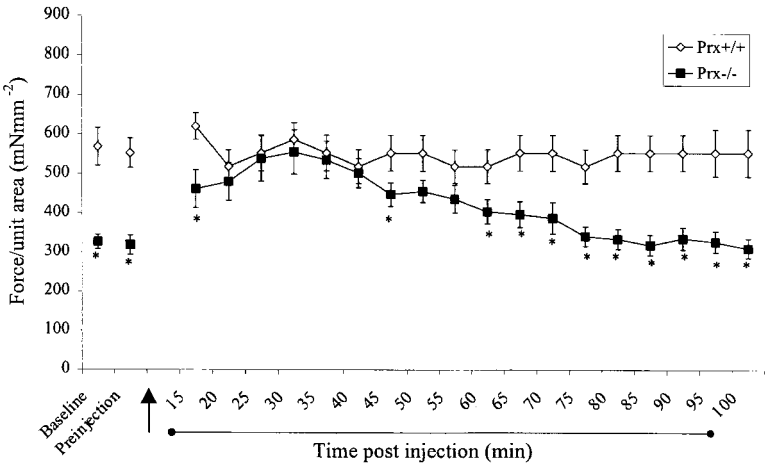

Figure 5. The Effect of Intrathecal Injection of (R)-CPP on Mechanosensitive Paw Withdrawal Threshold

The paw withdrawal threshold was measured in response to cutaneous mechanical stimulation with von Frey filaments in $Prx^{+/+}$ (n = 3) and $Prx^{-/-}$ (n = 8) mice after intrathecal injection of (R)-CCP (↑, 100 pmol in 10 μl). Statistical significance of differences from wild-type animals (*$p \leq 0.05$) was determined by Mann-Whitney Rank Sum test, and each value is the mean ± SEM. Intrathecal injection of the saline vehicle had no significant effect in either $Prx^{+/+}$ or $Prx^{-/-}$ (n = 5 for both).

Discussion

Several demyelinating neuropathies of genetic origin have now been modeled in mice by inactivating genes expressed in myelinating Schwann cells (Suter and Snipes, 1995; Martini, 1997). Such animals are not only valuable for examining the biological function of the proteins encoded by these genes but they also provide an opportunity to study the pathophysiology of disease. However, the homozygous periaxin null mouse is unique in the late onset and the severity of the clinical phenotype, which shows parallels with demyelinating peripheral neuropathies of adult onset (Dyck et al., 1993). Furthermore, sensory deficits have not been a significant feature of mouse models of peripheral demyelinating neuropathy thus far even though they are of relevance to human disease.

Prx Is Required for Stable Myelination in the PNS

This work suggests that the periaxin PDZ domain proteins have an essential role in myelin-forming Schwann cells once the sheath has been assembled around the axon. In *Prx* null mice, the earliest evidence for morphological disruption of the Schwann cell–axon unit is the focal thickening of the sheath. The ensuing overproduction of myelin is followed by demyelination and remyelination. The mechanism by which the thickness of the myelin sheath is signaled is not known, but it is likely that it is regulated by the axon (Lemke and Chao, 1988). It may be significant that the *Prx* gene itself is regulated by axonal contact (Scherer et al., 1995).

Many of the other well-characterized proteins expressed by differentiating, myelin-forming Schwann cells are incorporated as structural components of compact myelin. In contrast, L-periaxin becomes concentrated in the abaxonal plasma membrane of the Schwann cell, which suggested that it may recruit proteins to a cortical structure involved in transmembrane signaling (Gillespie et al., 1994; Scherer et al., 1995; Dytrych et al., 1998). The periaxins may associate with the cytoplasmic domains of plasma membrane proteins involved in adhesion since their expression coincides with the early events in the establishment of axo–glial contact and since the translocation of L-periaxin to a predominantly abaxonal location is a feature of the maturation of the sheath (Gillespie et al., 1994; Scherer et al., 1995; Dytrych et al., 1998).

Structure and Electrophysiology of the PNS in Mutant Animals

At 6 weeks of age, the sciatic nerves of periaxin-deficient mice are affected relatively mildly compared with mice lacking the myelin proteins P0 and PMP22, which display widespread derangements to the Schwann cell–axon unit from a much earlier age (Giese et al., 1992; Adlkofer et al., 1995). Mice lacking P0 also display clasping of their hindlimbs, but, at 4–6 weeks, they are much more clinically affected than are *Prx* mutants (Giese et al., 1992). This foot clasp response is also observed in mutant mice with neurological disease of purely CNS origin (Chiesa et al., 1998).

Similar to periaxin null mice, animal models of Charcot-Marie-Tooth disease Type X (CMTX) deficient in the gap junction protein connexin 32 myelinate normally and show a late onset pattern of demyelination with attempts

Table 4. Hindpaw Withdrawal Response to Noxious Thermal Stimulation following Intrathecal Administration of the NMDA Receptor Antagonist (R)-CPP in 6-Month-Old Mice

		Treatment		
	Preinjection	Vehicle	(R)-CCP	Recovery
Wild-type	9.1 ± 0.7 (5)	11.9 ± 1.6 (6)	10.0 ± 1.3 (5)	9.0 ± 1.9 (5)
Mutant	6.6 ± 0.3* (8)	9.1 ± 1.4* (6)	12.9 ± 1.3 (8)	6.7 ± 0.8* (8)

The effect of intrathecal injection of (R)-CCP (100 pmol in 10 μl) was measured as the mean threshold (latency to paw withdrawal) ± SEM of values determined between 20 and 50 min following injection and, at recovery, between 100 and 110 min following injection. Statistical significance from wild-type values with the same treatment (*$p \leq 0.05$) was determined by Student's t test. Number of animals is in brackets.

to remyelinate (Anzini et al., 1997). However, these animals display very mild deficits in nerve conduction; furthermore, the saphenous nerve, which is predominantly sensory, shows little evidence of demyelination in CMTX-deficient mice (Anzini et al., 1997). Mice heterozygous for the mutant alleles of P0 and PMP22 also show a propensity for sparing sensory versus motor nerves (Adlkofer et al., 1995; Martini et al., 1995). There was no evidence for such sparing in *Prx* mutants since the profiles of ventral and dorsal roots in cross section were similar. The reason for the differential susceptibility of sensory versus motor nerves in other demyelinating conditions remains unclear.

When deprived of their intimate association with axons during Wallerian degeneration, myelin-forming Schwann cells revert to a premyelinating phenotype (Bunge, 1993; Mirsky and Jessen, 1996). This transition is accompanied by the removal of myelin debris, the downregulation of genes that encode myelin proteins, and the initiation of Schwann cell proliferation (Poduslo and Windebank, 1985; Lemke and Chao, 1988; Scherer and Salzer, 1996). Reinnervation is normally accompanied by remyelination. However, the process of repair appears to be compromised in the sciatic nerves of periaxin null mice. Although the reason for this impairment is not clear, it is of relevance to the cumulative residual deficits caused by repeated mechanical compression of peripheral nerves in such diseases as hereditary neuropathy with liability to pressure palsy (Lynch and Chance, 1997).

By 6 months of age, conduction velocities in the sciatic and saphenous nerves of mutant animals were reduced to 44% and 33% of the respective normal values. This was entirely consistent with the extensive demyelination observed in a variety of myelinated nerves throughout the PNS. The frequent incidence of demyelinated axons engulfed by supernumerary Schwann cells was strikingly similar to the onion bulb pathology characteristically found in human CMT disease Type IA (CMTIA) (Dyck et al., 1993).

Demyelination, Allodynia, and Hyperalgesia

Damage to sensory nerves is linked to pain and excessive sensitivity to touch in human peripheral demyelinating disease (Devor, 1989; Pentland and Donald, 1994), and there is good evidence that the mechanical hypersensitivity observed in tactile allodynia is mediated by myelinated A fibers (Woolf, 1997; Koltzenburg, 1998). In a variety of models of central (spinal) sensitization, there is evidence that peripheral sensitization may be a significant contributor to neuropathic pain behavior (Koltzenburg et al., 1994; Woolf and Costigan, 1999). Nevertheless, we could find no evidence for significant changes in the stimulus–response relationship in either A or C fibers in $Prx^{-/-}$ mice with either cutaneous mechanical or thermal nociceptive stimulation in periaxin null animals; nevertheless, the spinal cord reflex responsiveness to this traffic may be affected by sensitization, especially at central sites. A contributor to central sensitization may be the presence of spontaneous activity in saphenous afferents. While repetitive C-afferent stimulation is normally required to bring about this state, in conditions of peripheral inflammation, it may be maintained or promoted by Aβ input (Woolf and Costigan, 1999).

Furthermore, our evidence that NMDA receptor–dependent events at a central site were essential for the phenomena of mechanical allodynia and thermal hyperalgesia behavior to become manifest in $Prx^{-/-}$ mice indicates that some form of central change, probably sensitization, is a feature of the phenotype. NMDA receptor–dependent central sensitization characteristically underpins the mechanical allodynia and thermal hyperalgesia seen in a variety of other models of chronic pain states (Mao, 1993; Bennett, 1994; Dickenson and Sullivan, 1987; Chaplan et al., 1997; Woolf and Costigan, 1999).

Ectopic repetitive firing has been proposed to be the origin of abnormal sensation in diseases characterized by segmental demyelination, particularly when there is no evidence for axonopathic changes (Rasminsky, 1981; Tal and Eliav, 1996). Demyelinated segments can certainly serve as foci for spontaneous multiple spikes or for those evoked by mechanical stimuli (Smith and McDonald, 1980; Calvin et al., 1982; Baker and Bostock, 1992). Nevertheless, the origin of tactile allodynia and neuropathic pain in human demyelinating disorders is still poorly understood (Ropper and Shahani, 1984; Carter et al., 1998). The periaxin-deficient mouse is a promising new model in which to address this problem.

Experimental Procedures

Generation and Characterization of $Prx^{-/-}$ Mice

The genomic locus was targeted by homologous recombination using a cassette comprising the human β-actin promoter (Frederickson et al., 1989) and the neomycin resistance (*neo*) gene flanked by 4.3 kb and 2.5 kb of strain 129 DNA. The cassette was inserted between the SmaI site in exon 6 and the SacI site in exon 7 and subcloned into the plasmid pGEM11Zf. The construct was excised from the plasmid by digestion with NotI and SalI and electroporated into the ES cell line CGR8, maintained in the absence of a feeder layer (Mountford et al., 1994). After selection in G418, the homologous recombinants were identified by Southern blotting using probes external to the construct. The targeted alleles were identified by the presence of a 7.9 kb fragment instead of a 9.6 kb when DNA was digested with SacI and probed with external probe I, and by a 7.4 kb fragment instead of 9.1 kb when DNA was digested with KpnI and probed with external probe II (Figure 1A). Two clones, 57 and 83, were injected into C57BL/6 blastocysts, and their progeny were typed by Southern blotting of tail DNA. Heterozygous offspring were backcrossed to the parental C57BL/6 strain before intercrossing at the F6 generations. Control animals were age- and generation-matched $Prx^{+/+}$ mice or $Prx^{+/+}$ littermates. Mice homozygous for the mutant allele were identified by Southern blotting as described previously (Dytrych et al., 1998).

RNA and Protein Analysis

RNA was extracted from the sciatic nerves of 16-day-old mice (Cathala et al., 1983), and 3 μg was resolved and probed with a cDNA fragment comprising bases 1132–1549 of mouse periaxin cDNA as described previously (Dytrych et al., 1998). L- and S-periaxin proteins in sciatic nerves from 3-month-old animals were detected with isoform-specific antibodies in Western blots as described previously (Dytrych et al., 1998).

Microscopy and Cell Counts

Mice were perfused intravascularly with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). Nerves were removed, fixed for 2 hr in the same fixative, postfixed in OsO_4, and embedded in araldite. Ultrathin sections were stained with uranyl acetate and lead citrate and examined on a Phillips BioTwin electron microscope. The following nerves were sampled: sciatic, tibial, lumbosacral and lumbar plexi, cervical and lumbar

nerve roots, saphenous, vagus, and trigeminal. Blocks of cervical and lumbar spinal cord were also examined. The sciatic nerves of anesthetized mice were crushed as previously described (Scherer et al., 1995). For light microscopy, 1 μm resin sections were stained with toluidine blue. For teased fiber preparation, sciatic nerves either were postfixed with 2% OsO_4 in 0.1 M sodium cacodylate buffer for 2 hr, dehydrated, infiltrated in Spurr medium, teased in Spurr embedding medium, and examined by bright-field microscopy or were teased in 0.1% Triton X-100 in phosphate-buffered saline, stained with TRITC-phalloidin (Sigma) (0.1 mg/ml) for 1 hr, and examined with a Leica TCS4D confocal microscope to visualize microfilaments. For cell counts on dorsal root ganglia, mice were perfused with neutral buffered formalin, and ganglia were embedded in paraffin after further immersion fixation in formalin overnight. Sections (8 μm) were stained with fast cresyl violet, and neurons with nucleoli in clear nuclei were counted in every fifth section.

Electrophysiology

Mice were anesthetized with 25% urethane i.p. and maintained at 36°C–37°C with a radiant heat lamp. The saphenous femoral nerve was exposed in the medial thigh and dissected from its associated vein and artery. Further dissection under liquid paraffin enabled the identification of afferent preparations comprising a small number of units. For fibers with conduction velocities above 2 ms^{-1}, the mechanosensitivity of individual sensory receptors with high sensitivity was measured in hairy and hairless skin using calibrated von Frey hairs. The mechanical threshold was defined as the pressure required to cause units to fire 50% of the time by direct application of von Frey filaments to identified receptive fields. The conduction velocity of single identified afferent fibers was determined using bipolar electrodes and the peripheral stimulus technique (Iggo, 1958). The cutaneous responsiveness of fibers with conduction velocities below 2 ms^{-1} was tested following isolation of few-unit preparations, and a focal heat stimulator was set to deliver a preset focal radiant heat stimulus to the receptive field at an intensity determined by a thermocouple for a preset period of 10–15 s (Sobair et al., 1997). Interstimulus intervals >10 min were allowed between applications to mitigate receptor sensitization. The CAP was measured over a length of sciatic nerve. The femoral length of the sciatic nerve was exposed in an isolated solder ring pool that was flooded with liquid paraffin. Bipolar recording electrodes were positioned on the sciatic nerve at the level of the femoral head, and bipolar stimulating electrodes were placed on the sciatic nerve in the pool, near the knee. A second pair of stimulating electrodes was positioned over the skin covering the tibial nerve at the ankle. The peak of the fastest conducting wave of the CAP was measured, providing a mean value for the Aα fibers. The electromyogram caused by activation of a single skeletomotoneuron was measured in the interosseus muscles with a concentric needle electrode using just-threshold levels of excitation at two locations in the sciatic nerve (Sharma and Thomas, 1974).

Behavioral Tests and Drug Treatment

The threshold for hindpaw withdrawal in response to graded mechanical stimulation was measured in conscious animals at 6 weeks of age with von Frey filaments, which provide a calibrated indentation pressure against the hairless skin of the hindpaws. The threshold response in this test was defined as the filament that causes foot withdrawal five times in every ten applications (Meyer et al., 1979; Chaplan et al., 1994). The time for hindpaw withdrawal in response to a quantified noxious thermal stimulus provided by an infrared beam to the hairless surface of the paws was measured (Hargreaves et al., 1988). Before determining the effect of an NMDA receptor antagonist, mice were tested daily over a period of 2 weeks prior to injection. A minimum of five measurements were made per animal. Mice were briefly anesthetized with halothane and oxygen after determining baseline measurements for mechanical allodynia and thermal hyperalgesia and were injected intrathecally at the L4 level of the spinal cord with 100 pmol of (R)-CPP in saline (10 μl). Testing commenced after a 15 min period of recovery from anesthesia. Mice were then tested every 5 min.

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